

A Research Note

ISOLATION AND IDENTIFICATION OF NITROSOPROLINE IN UNCOOKED BACON

INTRODUCTION

CONTRARY to sporadic reports of nitrosamines in other foods, N-nitrosopyrrolidine (NO-Pyr) has been found in approximately 80% of bacon samples tested after frying (Crosby et al., 1972; Fazio et al., 1973; Sen et al., 1973). Although concentrations of NO-Pyr as high as 108 $\mu\text{g/kg}$ (ppb) (Fazio et al., 1973) have been noted, the quantities usually found are in the range of 10–20 $\mu\text{g/kg}$. This, however, is a matter of concern since NO-Pyr has been found to be carcinogenic to some test animals (Druckrey et al., 1967; Greenblatt and Lijinsky, 1972a, b; Greenblatt et al., 1973). Nitrosopyrrolidine could be formed by the nitrosation of pyrrolidine, which may arise from the cyclization of putrescine (1,4-tetramethylenediamine) or from the decarboxylation of proline. Another possible pathway involves prior nitrosation of proline followed by decarboxylation to yield NO-Pyr. The decarboxylation of nitrosoproline (NO-Pro) has been reported recently (Bills et al., 1974; Fiddler et al., 1973; Pensabene et al., 1974) in bacon model systems under conditions similar to those for frying bacon. Nitrosopyrrolidine is found only in cooked bacon (Fazio et al., 1973; Sen et al., 1973; Pensabene et al., 1974). The concentration formed appears to be dependent on the amount of nitrite added during bacon curing (Sen et al., 1974), the bacon adipose tissue (Fiddler et al., 1974) and the temperature of cooking (Fiddler et al., 1973; Pensabene et al., 1974).

In this paper we are reporting the isolation and identification of NO-Pro in uncooked bacon and its possible role as the precursor for NO-Pyr.

EXPERIMENTAL

Materials

Putrescine, pyrrolidine and NO-Pyr were obtained from commercial sources. ^{14}C -labeled and unlabeled NO-Pro were synthesized in our laboratory from proline and NaNO_2 under acidic conditions similar to those reported previously (Pensabene et al., 1972). Each of these compounds was checked by thin-layer or gas-liquid chromatography, and no detectable impurities were found.

Formation of nitrosopyrrolidine in a model system

(a) **Putrescine.** 50 mg (5.7×10^{-4} moles) putrescine was added to 100 ml silicone oil (sp.gr. 0.93) (500 mg/l or ppm) heated for 6 min at 185°C. Hexane, 50 ml, was added to the silicone oil reaction mixture and extracted twice with 50 ml H_2O . To the combined aqueous extracts, 173 mg (2.5×10^{-3} moles) of NaNO_2 and 3 ml conc HCl were added and the solution heated at 52°C for 2 hr. The reaction mixture was extracted twice with CH_2Cl_2 , combined, washed with 50 ml 6N HCl, then 50 ml 5N NaOH, dried and concentrated to 1 ml.

(b) **Pyrrolidine.** 2 mg (2.8×10^{-5} moles) pyrrolidine was added to 100 ml H_2O (20 mg/l) and reacted with 173 mg NaNO_2 and 3 ml conc HCl as described in (a).

(c) **Nitrosoproline.** 10 μg (7.0×10^{-8} moles) NO-Pro was added to 100 ml silicone oil (100 $\mu\text{g/l}$ or ppb) and heated for 6 min at 185°C. Hexane, 50 ml, was added to the reaction mixture, then extracted twice with 50 ml H_2O . The aqueous extracts were combined and reextracted 3 times with 100 ml CH_2Cl_2 . The CH_2Cl_2 extracts were combined and washed with acid and alkali and concentrated as described above.

Isolation of nitrosoproline from bacon

A 100g sample of bacon was ground and blended twice with 200 ml cold distilled H_2O for 5 min. The homogenized material was centrifuged for 20 min at 10,000 rpm. The supernatants were decanted, filtered and made slightly alkaline (pH 7.5) with 0.5N NaOH, then refrigerated overnight to facilitate fat removal. The aqueous solution was extracted twice with 400 ml CH_2Cl_2 prior to freeze drying and the CH_2Cl_2 extracts discarded. Freeze-dried solids were extracted three times with 15 ml MeOH; the extracts were filtered, concentrated to 7 ml, centrifuged and then applied to 3g of 100 mesh silicic acid under nitrogen. The total material was transferred to a fritted glass thimble and continuously extracted with 50 ml redistilled ether in a Goldfisch extraction apparatus for 48 hr. Ether extracts were evaporated to dryness and the remaining solids esterified twice with 4 ml diazomethane in ether (prepared from Aldrich N-methyl-N-nitroso-p-toluenesulfonamide as directed) at room temperature for 15 min, the volume reduced to 1 ml, then subjected to GLC.

Determination of nitrosopyrrolidine in fried bacon

The fried bacon samples were analyzed for NO-Pyr using a modification (Fiddler et al., 1974) of the procedure described by Fazio et al. (1971).

Gas-liquid chromatography (GLC)

Nitrosopyrrolidine in CH_2Cl_2 extracts obtained from the analyses of fried bacon and from reaction mixtures was detected by GLC using an alkali flame ionization detector using conditions described previously by Pensabene et al. (1974).

Nitrosoproline methyl ester (NO-ProMe) was detected and quantitated using a Nuclear Chicago Model 5000 Gas Chromatograph equipped with a conventional flame ionization detector. The sample was separated on a 6 ft \times 1/8 in. o.d. stainless steel column packed with 6% Silar 10C on 60–80 mesh Gas Chrom P equipped for on-column injection. The gas flow conditions were: helium 40–, hydrogen 44– and air 400 ml/min. Injector port and detector temperatures were 190°C and 250°C, respectively. Nitrosoproline methyl ester had a retention time of 27.5 min (173°C) when the column temperature was programmed from 90 to 200°C at 3°/min.

Thin-layer chromatography (TLC)

An ether extract concentrate derived from silicic acid treatment containing the isolated NO-Pro was subjected to thin-layer chromatography prior to esterification. Silica gel plates, 250 micron, were used with butanol-acetic acid- H_2O (4:1:1). The plates were subjected to UV light then sprayed with Griess Reagent (sulfanilic acid and 1-naphthalenediamine) to give a pink colored spot having the same R_f value as NO-Pro standard.

GLC-mass spectrometric analysis

A Varian-Aerograph Model 1740-1 gas chromatograph equipped with a 6 ft \times 1/8 in. column packed with 6% Silar 10C on 60–80 mesh Gas Chrom P was interfaced with a DuPont Model 21-492 Mass Spectrometer. The GLC conditions used were identical to those described for the determination of NO-ProMe except for a helium carrier flow rate of 30 ml/min, and a temperature program of 120–200°C at 2°/min.

Nitrosopyrrolidine and NO-ProMe were identified by the coincident gas chromatographic retention time compared with the authentic nitrosamines and by high resolution mass spectrometry. The mass spectrometer was operated in the peak matching mode and adjusted to a resolution of 1 in 12,000 under the same conditions described previously (Pensabene et al., 1974). Nitrosopyrrolidine was confirmed by

Table 1—Nitrosamines in bacon

Sample	Raw nitrosoproline ^a (mg/kg)	Fried nitrosopyrrolidine ^a (μg/kg)
A	1.18	7
B	0.38	10
C	0.81	17

^a Confirmed by mass spectrometry

using its parent peak (m/e 100.06366) and m/e 99.99361 reference peak of perfluorokerosene (PFK). Nitrosoproline methyl ester was confirmed in a similar manner by determining m/e 158.069136 (parent peak) m/e 128.071148 (parent ion less the NO group) and m/e 99.055834 (nitrosopyrrolidine ion) using the appropriate PFK reference peaks.

RESULTS & DISCUSSION

THE POSSIBLE PATHWAYS for formation of NO-Pyr were investigated in model systems, and the quantity of precursor needed to yield 10 μg/l (ppb) NO-Pyr was determined. With putrescine as the starting material approximately 500 mg/l (ppm) was needed under conditions similar to those recommended for the frying of bacon. Since our laboratory had previously found that the highest putrescine content of lean tissue in green pork bellies was 36 ppm (Spinelli et al., 1974) it appears that this compound may have little, if any, role in the formation of NO-Pyr in bacon. The amount of pyrrolidine formed from putrescine in the model system was not determined. However, varying concentrations of pyrrolidine were nitrosated. It was found that 20 mg/l pyrrolidine would have to be present or be formed from putrescine in order to yield 10 μg/l NO-Pyr. The decarboxylation of NO-Pro is another possible pathway for NO-Pyr formation. It was found that 100 μg/l NO-Pro, heated at 185°C for 6 min, was needed to yield 10 μg/l NO-Pyr. This represented 14% yield theoretical quantity of NO-Pyr. These values coupled with the facts that the optimum temperature for this chemical reaction is about the same as that of normal frying conditions (Pensabene et al., 1974), and that proline, a weaker base than pyrrolidine, nitrosates more readily, suggested that NO-Pro might be the precursor of NO-Pyr in fried bacon. Analytical determination of NO-Pro in raw bacon was difficult because this compound is not volatile, it is unstable in acidic solution and on heating, and it undergoes rapid photolytic cleavage in aqueous solution by ultra-violet light derived from fluorescent lamps. With the aid of ¹⁴C-labeled NO-Pro the extraction and purification procedures described in the Experimental section were established. After freeze drying, 80–90% of the labeled NO-Pro was recovered, but large losses were encountered in the silicic acid clean-up procedure with only 20–25% of the original material accounted for. However, since the primary objective of the initial study was to determine the presence of NO-Pro in raw bacon, improvement in the procedure to increase the recovery was deferred. Samples of raw bacon were analyzed for NO-Pro prior to frying and NO-Pyr after frying at standard conditions. Results in Table 1, confirmed by mass spectrometry, show

that NO-Pro was present in the uncooked bacon at concentrations ranging from 0.38–1.18 mg/kg, uncorrected for recovery, whereas the NO-Pyr in the fried bacon occurred at 7–17 μg/kg.

The qualitative presence of NO-Pro in bacon has been described. This nitrosamine has been found to be noncarcinogenic by Garcia and Lijinsky (1973); Greenblatt and Lijinsky (1972a). This is the first report of a nonvolatile nitrosamine found in a food product. The possibility exists that other food products, particularly cured meats, which contain proline and nitrite, also have NO-Pro present. In addition, other nonvolatile nitrosamines, potentially deleterious to the health, may be formed.

Cautionary note

It should be remembered that many nitrosamines are potent carcinogens to test animals. Proper precautions against inhalation of the vapors and contact with the skin should be maintained.

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